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Abstract

Patients with recessive dystrophic epidermolysis bullosa (RDEB) frequently present with squamous cell carcinomas (SCCs) probably as a result of chronic blistering and extensive scarring. These tumors are clinically aggressive as they metastasize readily. The metastasis-associated protein (MTA)-1, a transcription suppressor, is overexpressed in several epithelial neoplasms including SCCs. Our preliminary results demonstrate that MTA1 expression is induced by activation of the epidermal growth factor receptor (EGFR). As deregulation of EGFR signaling is frequently observed in aggressive epithelial neoplasms we propose to study the role of EGFR signaling and MTA1 expression in SCCs derived in RDEB patients. Our Specific Aims are to establish cell lines derived from SCCs in non-RDEB and RDEB patients, characterize the malignant phenotype of these cells as it relates to EGFR expression and signaling and to expression of MTA1, examine the contribution of EGFR/MTA1 to proliferation, invasiveness, and cell survival and identify EGFR-dependent signaling pathways contributing to MTA1 expression in these cells. The results from this research will provide invaluable tools for future analysis of the pathobiology of carcinoma cells and will ascertain whether EGFR/MTA1 signaling pathways contributes significantly to the metastasis and invasiveness of SCC derived from RDEB patients.

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INTRODUCTION

Epidermolysis bullosa (EB) is a group of heritable genetic skin blistering diseases caused by the disruption of the normal function of the basement membrane zone (1,2). Skin blisters between the dermal/epidermal junction in EB are extremely similar to those caused by chemical warfare agents including sulfur mustard (dichlorodiethyl sulfide). Mutations in the collagen type VII gene that encodes for type VII collagen, an epidermal basement membrane zone protein, is linked to the recessive dystrophic forms of EB (RDEB) (3-6). As a result of chronic blistering, re-epithelialization, and extensive scarring, RDEB patients frequently develop highly invasive squamous cell carcinomas (SCC) (7-11). SCC is the second most common form of skin cancer with approximately 250,000 new cases in the United States yearly. SCCs are generally associated with chronic ultraviolet light exposure, but they can also arise in association with persistent skin wounds as in the cases of RDEB. More than 55% of RDEB patients die from SCC by age 40 due to the highly metastatic and invasive properties of the RDEB-SCCs. Recent exciting findings that fibronectin-like sequences within NC1 domain of collagen VII were required for tumorigenesis and that RDEB patients with retention of this domain may be more susceptible to squamous cell carcinomas (12). Despite the high frequency of these neoplasias in RDEB patients, very little is known about the molecular characteristics of these tumors in comparison to SCCs occurring spontaneously in individuals without EB.

The metastasis tumor antigen 1 (MTA1) belongs to the large family of metastasis-associated genes (MTAs) including three different members (MTA1, MTA2, and MTA3) and six reported isoforms (MTA1, MTA1s, MTA1-ZG29p, MTA2, MTA3, and MTA3L). MTA1, along with MTA2 and MTA3, are members of the nucleosome-remodeling and transcription regulation histone-deacetylase (NuRD) complex (for review see 13). MTA1 is overexpressed in and correlates well with many highly metastatic cancers including human epithelial-derived breast and esophageal carcinomas (14-17). We previously demonstrated that MTA1 is overexpressed in SCCs relative to normal keratinocytes (18). Furthermore, we found that MTA1 expression is controlled by the activation of the epidermal growth factor receptor (EGFR) in this cell type. Forced overexpression of MTA1 in keratinocytes increases cell proliferation and invasiveness, characteristics evident in SCCs from RDEB.

The overall goal of our research through DAMD17-02-1-0215 is to begin an in depth investigation of RDEB-derived SCCs and ascertain whether the epidermal growth factor receptor- (EGFR) and/or MTA1 mediated signaling pathways contribute significantly to the highly aggressive malignant phenotype these cells. In order to study the molecular characteristics and mechanisms of metastasis in these cancer cells, we proposed to establish cell lines derived from SCC of RDEB patients and to characterize the malignant phenotype (growth, invasiveness, survival potential, and dependence on EGFR/MTA1 activation) of these cells. Our **Specific Aim 1** is to establish and characterize epithelial keratinocyte SCC cell lines from RDEB and non-RDEB skin biopsies. **Specific Aim 2** is to characterize the malignant phenotype (growth, invasiveness, and survival potential) of these cells as it relates to EGFR expression and signaling and to expression of MTA1. **Specific Aim 3** is to examine the contribution of EGFR/MTA1 to proliferation, invasiveness, and cell survival. Finally, **Specific Aim 4** is to identify the signaling pathways downstream of EGFR activation that are relevant to MTA1 expression in SCC from RDEB and non-RDEB.

BODY**Deregulated Signal Transduction Pathways in the Epidermis of RDEB-SCC**

An issue of critical importance is whether signaling events are deregulated in RDEB epidermis *in situ*. We assessed activation of signaling pathways commonly implicated in epidermal hyperplasia and tumor development, i.e. the PI-3-kinase/AKT and the Raf/MEK/MAPK pathways (**Fig. 1**). This analysis revealed some significant results. First we observed no major changes in levels of Protein Kinase B (AKT) phosphorylation on both Ser473 and Thr308 (not shown) and of the AKT target GSK-3 β on Ser9 in RDEB-SCCs as compared to normal skin. Interestingly, AKT-Thr308 is phosphorylated by the 3-Phosphoinositide-Dependent Protein Kinase (PDK)1 (for review see 19) which showed enhanced phosphorylation in RDEB-SCCs. Finally, we examined Ser308 phosphorylation of PTEN, a negative regulatory phosphatase of the PI-3-kinase/AKT pathway. Again, we observed no significant changes in the phosphorylation levels of PTEN. Collectively, these results reveal little if any activation of AKT-dependent signaling events in RDEB-SCC. When we examined the Raf/MEK/MAPK axis a more complicated picture emerged. While Raf phosphorylation was not detected in any tissue and the phosphorylation state p42/44MAPK was not changed, the phosphorylation of MEK1/2 was greatly enhanced in extracts from RDEB-SCCs. Unexpectedly, however, the MAPK substrate p90^{RSK}, but not ELK1, revealed higher levels of phosphorylation in RDEB-SCC raising the question whether phosphorylation of p90^{RSK} was induced by kinases other than p42/44MAPK. p90^{RSK} is most likely phosphorylated by PDK1 but possible involvement of stress-activated MAP kinases (p38MapK or JNK1/2) in this phenomenon is currently being examined. Finally, we observed elevated level of c-Myc and Mad1 in RDEB-SCCs. The Myc/Max/Mad1 signaling pathway regulates cell proliferation, migration, differentiation, and apoptosis. It appears that this Myc/Max/Mad1 proteins function as transcriptional regulators in the progression of RDEB-SCC. In addition, Myc/Max/Mad1 are also implicated in the regulation of the human telomerase reverse transcriptase (hTERT) gene through acetylation/deacetylation of histones (20). We observed MTA1 upregulation in RDEB-SCCs. MTA1 is a member of the NuRD complex and enhances the NuRD's nucleosome remodeling activity. Collectively, a complex picture emerges intricately linking the growth factor/mitogen activated signaling pathways and MTA1 involvement in RDEB-SCCs.

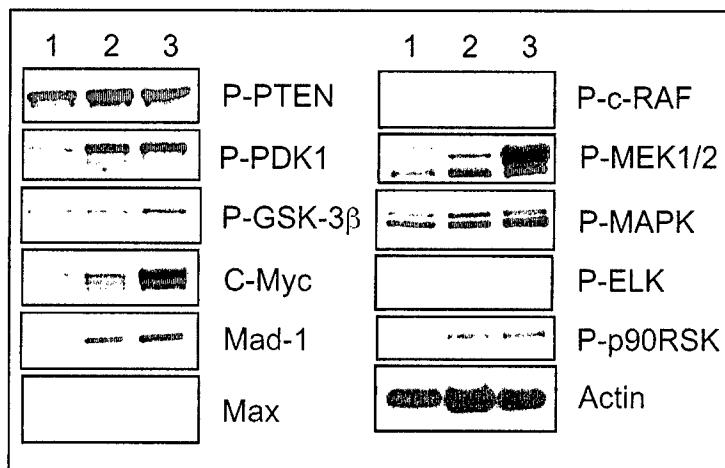
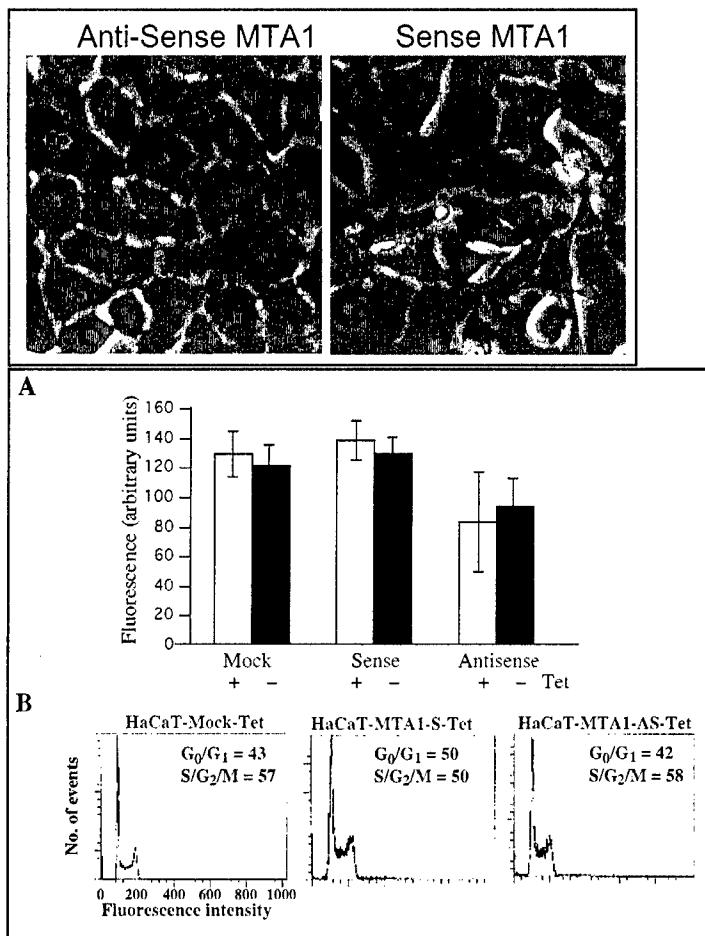


Fig. 1 Characterization of the signaling events in RDEB SCCs. Total protein lysates from normal human skin (lane 1) and RDEB SCCs (lanes 2 and 3) were analyzed by Western blotting analysis with a panel of antibodies from Cell Signaling Technology. Loading control was actin.

Cell Cycle Regulation by MTA1

In order to assess the role of MTA1 in epithelial cell proliferation, we established variants of human immortalized keratinocytes (HaCaT cells) by expressing MTA1 cDNA in both the sense and antisense orientations (18). Further characterization of these epithelial cells lacking MTA1 demonstrated a reduction in cell cycle progression and appeared morphologically similar to quiescent non-proliferating basal cells (**Fig. 2**). To determine effects of MTA1 expression on cellular metabolism, HaCaT-Mock, HaCaT-MTA1-S (sense), and HaCaT-MTA1-AS (antisense) cells were grown for two days in the absence of tetracycline to induce transgene expression and then treated with Alamar Blue™. As determined by this assay overexpression of either sense or antisense MTA1 sequences had no significant effect on cellular metabolism (**Fig. 3A**). However, reduced cellular metabolism (consistent with reduction in cell proliferation) was observed in the cells overexpressing the antisense sequence (**Fig. 3A**). On the other hand, while the expression of the antisense MTA1 sequence did not detectably affect cell cycle distribution as assessed by FACS analysis of propidium iodide stained cells, expression of the sense MTA1 sequence reduced the number of cells in S/G₂/M driving those cells through mitosis into the G₀/G₁ stage of the cell cycle (**Fig. 3B**).



the percentage of cells in G₀/G₁ and decreased the percentage of cells in S/G₂/M. Note the absence of apoptotic cells with sub-G₀/G₁ DNA content.

We previously demonstrated that MTA1 expression is necessary but not sufficient for cell survival in the anchorage independent state. Here we designed experiments to demonstrate the effects of MTA1 on keratinocyte cell survival by determining cell death in suspension culture. MTA1 sense and antisense cells were put in suspension culture with EGF or mAb425 (inhibitor of EGF receptor). Cells were then subjected to flow cytometric analysis of terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL)-positive cells. Apoptotic changes was measured by the appearance of TUNEL-positive cells (**Fig. 4**). The percentage of cell death was similar between cells expressing MTA1 sense or antisense. By contrast, EGF provided some protection from cell death in the MTA1 sense but not antisense expressing cells. Anoikis was markedly increased in both MTA1 sense and antisense cells treated with mAb425. Collectively, these results demonstrate that MTA1 expression contributes to several aspects of the metastatic phenotype including enhanced cellular proliferation and survival in the anchorage independent state.

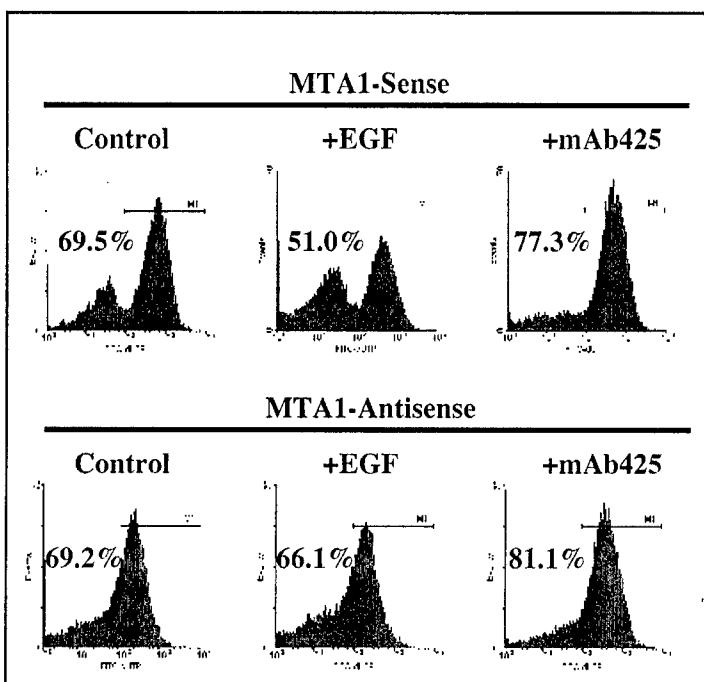


Fig. 4. EGFR-dependent survival and viability of epithelial cells expressing MTA1 sense or antisense. TUNEL staining of HaCaT cells overexpressing MTA1 (sense or antisense sequences) in the presence of EGF (10 ng/ml) or mAb425 (10 µg/ml); values represent the cell fractions stained with FITC-dUTP relative to controls (HaCaT-mock).

It has been documented that hypoacetylation of histones favors transcriptional silencing, a potential underlying cause of cell proliferation and cancer development. MTA1 has been shown to interact directly with and enhance the histone deacetylase activity of HDAC1/2 in the NuRD complex. We previously demonstrated that MTA1 regulates growth, invasiveness, and survival potential of normal epidermal keratinocytes. Here we report a novel role for MTA1 in regulating cellular processes, that when go awry, would favor carcinogenesis. MTA1 has been shown to interact with MICoA and endophilin 3 and modulate the transactivation functions of ER-a. By yeast two-hybrid protein-protein interaction system and in vitro or in vivo binding assays, we observed the direct interaction between MTA1 and REV7 (UV revertible gene) and MAD2 (mitotic arrest-deficient) (Fig. 5). Rev7, along with Rev1 and Rev3, make up the polymerase zeta (Pol zeta) complex, an error-prone DNA polymerase. In yeast, Pol zeta is responsible for spontaneous as well as ultraviolet radiation-induced mutagenesis (21). B-lymphocytes devoid of any of the Pol zeta proteins died from chromosomal instability and hypersensitivity to various genotoxic treatments. More interestingly, in human REV7 interacts with the mitotic spindle checkpoint protein MAD2 (22). MAD2 accumulates on kinetochores in early mitosis and relocates to the spindles during anaphase (for review see 23). Thus, it has been proposed that REV7 and hMAD2 might control cell cycle progression into anaphase by regulating the activation of the anaphase promoting complex. Thus, it is only befitting that cells lacking MTA1 would show decrease in cell proliferation (Figs. 2&3) and epithelial carcinomas overexpressed MTA1 as well as MAD2 (Fig. 6). One interesting recent observation was that cells lacking MTA1 showed increase in MAD2 protein suggesting that MTA1 may regulate MAD2 expression (Fig. 7). In order for MTA1 to interact with REV7 or MAD2, it must colocalize with these proteins, particularly during mitosis when they are active. In non-mitotic interphase cells, MTA1 was found localized to the nuclei (Fig. 8A). However, during division as cells undergo mitosis when MAD2 has been shown to bind to the kinetochores of chromosomes, MTA1 was detected co-localized to microtubule spindles (Fig. 8B&9). Collectively, these results demonstrate the diverse roles of MTA1 from nucleosome remodeling by enhancing histone deacetylation to DNA repair and spindle assembly checkpoint during mitosis.

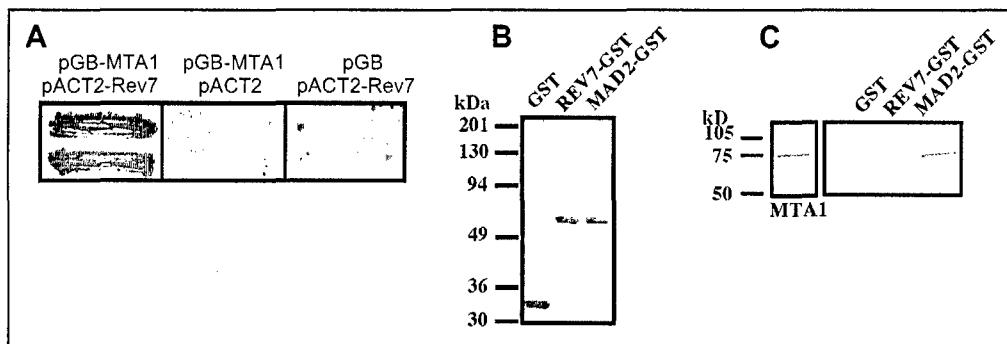


Fig. 5. Molecular interaction of MTA1 with REV7 and MAD2 and potential role of MTA1 during mitosis. (A) Yeast two hybrid system revealed interaction between the MTA1 bait and the REV7 protein. (B) We generated bacterially produced recombinant glutathione S transferase fusion proteins of REV7 and MAD2. Western blotting with anti-GST antibodies. (C) GST pull down assay to demonstrate that MTA1 interacted with REV7-GST and MAD2-GST but not GST alone.

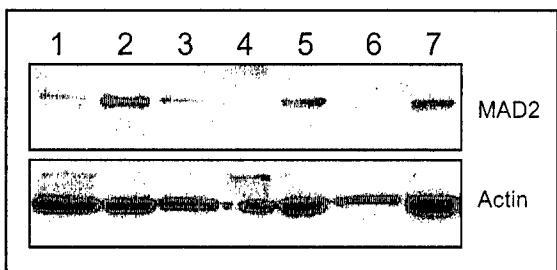


Fig. 6. Expression of MAD2 normal and SCC cells. Western blotting analysis of MAD2 in various cell lines. 1, HaCaT; 2, A431; 3, FaDu; 4, SCC12; 5, SCC13; 6, SiHa; 7, Cos-7 (SV40-transformed African Green monkey kidney).

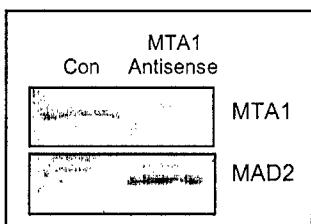


Fig. 7. MTA1 regulates MAD2 expression. Western blotting analysis of HaCaT cells expressing MTA1 antisense with MTA1 and MAD2 antibodies. MTA1 protein level decreased while MAD2 increased in cells expressing the MTA1 antisense.

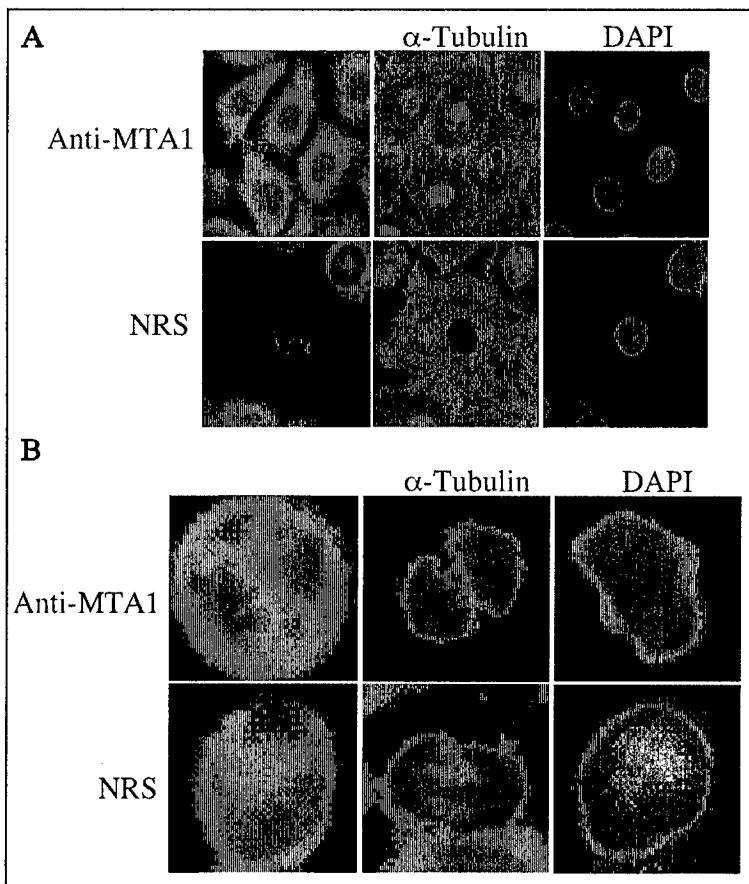


Fig. 8 MTA1 is localized to the nucleus in interphase but associated with microtubules during mitosis.
Immunofluorescence analysis of MTA1 (red) and α -tubulin (green) in quiescent (A) and mitotic (B) HaCaT cells. DNA was labeled with DAPI.

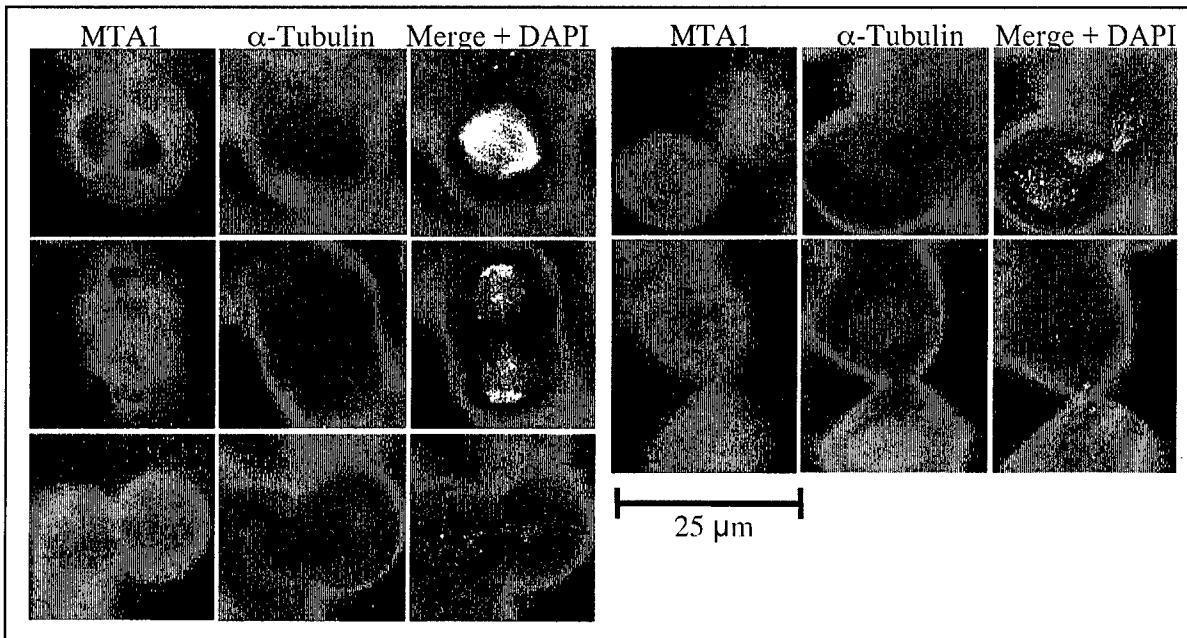


Fig. 9 Subcellular Distribution of MTA1. Immunofluorescence analysis of MTA1 (red) and α -tubulin (green) in HaCaT cells during metaphase, anaphase, various stages telophase, and cytokinesis. DNA was labeled with DAPI. Bars, 25 μ m.

In order to study the biology of tumor cells from RDEB, we had proposed to establish these cells in culture. This is a critical part of this proposal because these cells will serve as excellent tools for any future studies involving keratinocyte tumor biology. Within this year, we collected skin RDEB-SCCs but were unsuccessful at maintaining these cells in culture. The reasons are unclear and are being resolved. Due to this technical problem, we have obtained the help from two investigators, Mei Chen (Division of Dermatology, University of Southern California, Los Angeles CA) and M. Peter Marinkovich (Department of Dermatology and Epithelial Biology; Stanford University School of Medicine, Stanford CA). These investigators recently sent us RDEB cell lines as described in their recent publications (12,24). We are currently maintaining and characterizing these cells.

KEY RESEARCH ACCOMPLISHMENTS

- We made several exciting new findings this year with the first showing evidence of deregulated signal transduction pathways in the epidermis of RDEB-SCC.
- Secondly, we have further characterized the role of MTA1 in DNA repair and mitotic spindle assembly checkpoint. The results provided are still preliminary and we plan on further assessing the mechanism regulating this interaction and the role of EGF receptor signaling pathway in regulating this interaction during year 4 of this funded research.

REPORTABLE OUTCOMES

No reportable outcome at this point. However we hope to have a manuscript describing these exciting new findings completed in the near future.

CONCLUSIONS

During the third year of this funding we have collected RDEB tumor tissues and although we were unable to establish tumor cell lines derived from these samples, we have obtained some cell lines from other investigators. Our initial characterization of the signaling pathways (*in vivo*) commonly implicated in epidermal hyperplasia and tumor development, i.e. the PI-3-kinase/AKT and the Raf/MEK/MAPK pathways. We will complete this analysis during the forth year of this proposal.

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APPENDIX

None